Medical Microbiology and Immunology

GENERATION, MAINTENANCE AND TISSUE DISTRIBUTION OF T CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS IN LYTIC AND LATENT INFECTION.

--Manuscript Draft--

Manuscript Number:	MMIM-D-19-00031R1
Full Title:	GENERATION, MAINTENANCE AND TISSUE DISTRIBUTION OF T CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS IN LYTIC AND LATENT INFECTION.
Article Type:	Review
Keywords:	Human Cytomegalovirus (HCMV); T cell memory; Memory inflation; Latency
Corresponding Author:	Sarah Elizabeth Jackson, Ph.D. University of Cambridge Cambridge, Cambridgeshire UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Cambridge
Corresponding Author's Secondary Institution:	
First Author:	Sarah Elizabeth Jackson, Ph.D.
First Author Secondary Information:	
Order of Authors:	Sarah Elizabeth Jackson, Ph.D. George X Sedikides Georgina Okecha Mark Ralph Wills
Order of Authors Secondary Information:	
Funding Information:	Medical Research Council (MR/K021087) — Dr Mark Ralph Wills Medical Research Council (MR/S00081x/1) — Dr Mark Ralph Wills
Abstract:	Understanding how the T cell memory response directed towards human cytomegalovirus (HCMV) develops and changes over time while the virus persists is important. Whilst HCMV primary infection and periodic reactivation is well controlled by T cell responses in healthy people, when the immune system is compromised such as post-transplantation, during pregnancy, or under developed such as in new-born infants and children, CMV disease can be a significant problem. In older people HCMV infection is associated with increased risk of mortality and despite overt disease rarely being seen there are increases in HCMV DNA in urine of older people suggesting that there is a change in the efficacy of the T cell response following life-long infection. Therefore, understanding whether phenomenon such as “memory inflation” of the immune response is occurring in humans and if this is detrimental to the overall health of individuals would enable the development of appropriate treatment strategies for the future. In this review we present the evidence available from human studies regarding the development and maintenance of memory CD8+ and CD4+ T cell responses to HCMV. We conclude that there is only limited evidence supportive of “memory inflation” occurring in humans and that future studies need to investigate immune cells from a broad range of human tissue sites to fully understand the nature of HCMV T cell memory responses to lytic and latent infection.

UNIVERSITY OF CAMBRIDGE
Department of Medicine

Dr Mark Wills and Dr Sarah Jackson
Division of Infectious Diseases
Our Ref: MMIM-D-19-00031

Professor Matthias J. Reddehase
Editor in Chief
Medical Microbiology and Immunology

Dear Matthias

Ref.: Ms. No. MMIM-D-19-00031 - GENERATION, MAINTENANCE AND TISSUE DISTRIBUTION OF T CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS IN LYTIC AND LATENT INFECTION.

Thank you for your kind words about our manuscript, we have now revised the review in response to the comments made by yourself and the reviewer. Below we have provided a point by point response to each of the comments below, with our response to each point in blue type. The revised manuscript has incorporated the reviewers' comments as appropriate and the changes have been highlighted by line number of the revised document in this letter.

Response to comments:

(1) I appreciate your attention to the mouse model on p.3, line 78 pp and the correct reference to the work by Holtappels et al (Ref. 25) who were first to show memory inflation in the year 2000 in JVI. With knowledge of this work, Klenerman in 2003 (Ref. 26) expanded on the previous work by Holtappels and arrived at the same conclusions, coining the catch phrase "memory inflation" but not citing Holtappels et al. for the first description. The result is that mostly only Karrer et al. is cited for having first described memory inflation.

Please note that a debate at that time resulted in a published Erratum, which should be cited in the reference list along with the paper.

Memory Inflation: Continous Accumulation of Antiviral CD8+ T Cells Over Time. Urs Karrer, Sophie Sierro, Markus Wagner, Annette Oxenius, Hartmut Hengel, Ulrich H. Koszinowski, Rodney E. Phillips and Paul Klenerman. J Immunol October 1, 2003, 171 (7) 3895; DOI: https://doi.org/10.4049/jimmunol.171.7.3895-b

This reference has now been added to the text. [Revised document reference no.: 27]

Level 5, Box 157
Addenbrooke's Hospital
Hills Road
Cambridge CB2 2QQ

Tel: +44 (0) 1223 336862
Fax: +44 (0) 1223 336846
Email: mrw1004@cam.ac.uk
sej47@cam.ac.uk

www.med.cam.ac.uk/wills/

UNIVERSITY OF CAMBRIDGE
Department of Medicine

Actually, Ref. 25 was first to show

- Accumulation of IE1-specific CD8 T cells during latent infection
- Epitope-specificty in that IE1 is "inflationary" whereas others are non-inflationary, namely m04, M83, M84
- Accumulation in ABSOLUTE CELL NUMBERS, not just % (see comments by Reviewer)
- Effector-memory phenotype CD62L-minus
- Accumulated cells are functional in that they secrete IFN-gamma upon stimulation with peptide.

We have amended the text where the Holtappels et al 2000 reference is first cited to reflect these points. [Revised document line nos.: 89 - 91]

(2) In the list of epitopes that drive memory inflation m164 is missing. This actually was the second inflationary epitope identified in 2002 (still earlier than Karrrer et. al.2003) You already have this as reference Ref. 32 but cited it for IE1 only . Actually in Ref. 32 Holtappels et al. have identified the sequence of the m164 peptide and showed that this new peptide behaves inflationary like IE1.

We have amended the text where the murine epitopes that drive memory inflation are discussed to include m164 and the Holtappels et al 2016 reference. [Revised document line no.: 96]

(3) Regarding "continous inflation" I would like to draw your attention to the fact that this is idealized by selecting data that fit. The truth is that the dynamics is higher, with specificities rising and falling. See the recent paper in Frontiers in Immunology. Holtappels et al. 2016.

Thank you for this comment and bringing the Holtappels et al 2016 paper to our attention, we have added a reference to this observation to our discussion of fluctuations in the magnitude of HCMV responses over time. [Revised document line nos.: 168 - 169]

Let me also comment on some comments of the Reviewer (see below), as I disagree with the Reviewer in some points:

(1) " Authors would greatly help the field if they could consistently point out which, if any, of the studies actually followed absolute cell numbers"

I agree but would like to note that the first description by Holtappels in 2000 (Ref. 25) already showed memory inflation as increase in % IE1-specific among CD8s as well as increase in absolute numbers!

As well as discussing the increase in absolute numbers in the murine model in our introductory paragraph to the main text of the review [revised document line nos.: 87 - 91]. We have clarified which of the cited human studies used absolute numbers in their observations [revised document line nos.: 113 – 114; 119; 120 – 121; 130 – 132; 137; 169 – 174; 177 – 180; 201 – 204; 209; 213]. Furthermore we have added a small statement reflecting the importance of knowing the size of the immune cell compartment compared to frequencies [revised document line nos.: 121 - 124].

(2) "response being broader in B6 and humans than in BALB/c" . The reviewer also had m164 not in mind (see above) and other peptides in BALB/c are intermediate in inflation. This is not a real difference to B6 with 4 inflationary epitopes. Further, the famous paper by Sylvester (JEM) with the "broad response" in humans in the title is mostly misunderstood. The response is broad on the polymorphic population level with high HLA coverage. Sylvester have nicely shown that the response in INDIVIDUALS is less broad with a median of 8, as far as I recall. Considering that humans are not homozygous, unlike mouse inbred strains, there is no longer any difference between humans, BALB/c mice and C57Bl/6 mice.

In light of your comments and the other revisions we have made to the text we have decided to not to reference mouse strain differences highlighted by this reviewer, and have removed the reference to the size of the CMV specific response in BALB/c mice.

Despite my lengthy comment, actual changes will be a minimal revision, except if you wish to revise your British English. Please resubmit as fast as possible so that I can forward it to the publication Office.

Thank you for this comment, we have taken the opportunity to revise the overall text to hopefully improve the readability of the review. As this editing has occurred throughout the document we have not highlighted all of the changes here or in the main revised document. We have revised all sections of the text, including the abstract and the main body, hopefully the English language used is less cumbersome now.

Reviewers' comments:

Reviewer #1: The review by Jackson, Wills and colleagues addresses an important topic in CMV biology, asking critical questions related to the existence, or not, of HCMV-mediated memory inflation in the human immune system. The article is generally well-written. To improve it further, authors should revise the review to address the points below:

* The major conceptual issue in this review is that most, if not all, reviewed studies on memory inflation have dealt only with fractions and representation of different populations. Authors would greatly help the field if they could consistently point out which, if any, of the studies actually followed absolute cell numbers, and if they could highlight the importance of this parameter to distinguish absolute accumulation of different cell subsets/antigen-specific populations.

As mentioned above in response to your comment we have amended the text to show which studies used absolute count numbers versus frequency and also added a short statement regarding the importance of this parameter in human studies. [Revised document line nos.: 113 – 114; 119; 120 – 121; 121 – 124; 130 – 132; 137; 169 – 174; 177 – 180; 201 – 204; 209; 213]

Minor comments:

* Line 83, "20% of the CD8 response specific to one epitope of MCMV EI1", should note that in this model (BALB/c) EI1 is extremely immunodominant in both the acute and latent phases, unlikely in B6 mice or humans, where the response is much broader.

UNIVERSITY OF CAMBRIDGE
Department of Medicine

We have removed this line from the document, particularly as the earlier Holtappels et al 2000 publication are discussed in greater detail than before. [Revised document line nos.: 87 – 94]

* Line 126 - what is the "lytic latent protein group" vs the "latent protein group"? Or was that a typo? Regardless, it seems that it would be more precise to name them "epitopes (or peptides) belonging to lytic or latent protein groups"?

We have edited and amended the text to better clarify this section of the review. [Revised document line nos.: 141 – 145]

- Lines 70-72 : CD4 T cells cannot "…secrete IL-10 as well as antiviral effector functions". Please re-formulate. Subsequent sentence (lines 72-74) - should be edited for clarity, would better convey meaning if it had "in contrast" rather to "and a contrast".

We have edited this section to clarify the point being made. [Revised document line nos.: 75 – 80]

- Line 102, suggest to break the sentence in two; as is, the connector joins two separate thoughts.

We have edited this sentence as suggested. [Revised document line nos.: 110 – 114]

- Line 142-144, the sentence is run-on and difficult to follow, please break the sentence and provide references for each point.

We have edited this sentence as suggested and improved the referencing for clarity. [Revised document line nos.: 161 – 165]

- Line 201, discordance in singular/plural.

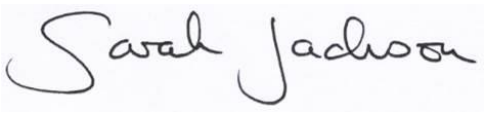
We have edited this sentence to improve understanding. [Revised document line nos.: 229 – 232]

- Lines 332-336, three sentences in one.

We have edited this sentence to improve the readability of this part of the review. [Revised document line nos.: 379 – 383]

We hope that the revisions we have made to the manuscript are suitable and meet your recommendations. We have also added the statement regarding the issue to the title page and the conflict of interest statement after the acknowledgments as requested by your email of the 2nd March [Revised document line nos: 1; 28 – 29].

Yours sincerely,



UNIVERSITY OF CAMBRIDGE
Department of Medicine

Dr Mark Wills & Dr Sarah Jackson

This article is part of the Special Issue on Immunological Imprinting during Chronic Viral Infection.

TITLE:

GENERATION, MAINTENANCE AND TISSUE DISTRIBUTION OF T CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS IN LYTIC AND LATENT INFECTION.

AUTHORS:

Sarah E. Jackson[a1], George X. Sedikides[a], Georgina Okecha[a] and Mark R. Wills[a1]

AFFLIATION:

[a] Division of Infectious Diseases, Department of Medicine, University of Cambridge, Level 5, Box 157, Addenbrookes Hospital, Hills Road, Cambridge, CB2 0QQ, United Kingdom.

[1] Joint corresponding author contact details:

Mark Wills, Division of Infectious Diseases, Department of Medicine, University of Cambridge, Level 5, Box 157, Addenbrookes Hospital, Hills Road, Cambridge, CB2 0QQ, United Kingdom. Tel: +44 1223 336862. Email: mrw1004@cam.ac.uk

Sarah Jackson, Division of Infectious Diseases, Department of Medicine, University of Cambridge, Level 5, Box 157, Addenbrookes Hospital, Hills Road, Cambridge, CB2 0QQ, United Kingdom. Tel: +44 1223 763402. Email: sej47@cam.ac.uk

ORCID IDs:

Sarah Jackson: 0000-0002-4230-9220
Mark Wills: 0000-0001-8548-5729

ACKNOWLEDGMENTS

We thank Dr Emma Poole, Martin Potts, Sara van den Berg and Eleanor Lim for contributing to the experimental work discussed in this review. We thank Prof. Paul Griffiths for useful discussions about the work presented in this review. We thank the Medical Research Council (MRC:UKRI) for funding [MR/K021087; MR/S00081X/1]. This research was supported by the Cambridge NIHR BRC cell phenotyping hub.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare they have no conflict of interest

ABSTRACT (227 words)

Understanding how the T cell memory response directed towards human cytomegalovirus (HCMV) develops and changes over time while the virus persists is important. Whilst HCMV primary infection and periodic reactivation is well controlled by T cell responses in healthy people, when the immune system is compromised such as post-transplantation, during pregnancy, or under developed such as in new-born infants and children, CMV disease can be a significant problem. In older people HCMV infection is associated with increased risk of mortality and despite overt disease rarely being seen there are increases in HCMV DNA in urine of older people suggesting that there is a change in the efficacy of the T cell response following life-long infection. Therefore, understanding whether phenomenon such as “memory inflation” of the immune response is occurring in humans and if this is detrimental to the overall health of individuals would enable the development of appropriate treatment strategies for the future. In this review we present the evidence available from human studies regarding the development and maintenance of memory CD8+ and CD4+ T cell responses to HCMV. We conclude that there is only limited evidence supportive of “memory inflation” occurring in humans and that future studies need to investigate immune cells from a broad range of human tissue sites to fully understand the nature of HCMV T cell memory responses to lytic and latent infection.

KEYWORDS

Human Cytomegalovirus (HCMV); T cell memory; inflation; latency.

REVIEW TEXT:

Introduction

Primary infection with human cytomegalovirus (HCMV) in healthy individuals does not generally cause overt disease [1,2], however a robust immune response is generated including neutralising antibodies and cellular responses which eventually controls and eliminates the lytic virus [3]. In the face of this immune response the virus is not cleared probably due to the numerous viral immune evasion proteins encoded by the virus [4,5] and is able to establish a latent infection in certain cell types [6]. Periodically the virus reactivates, resulting in antigenic stimulation of HCMV specific secondary immune responses and generating distinct memory CD4+ and CD8+ T cell populations, characteristic of this infection (recently reviewed in [7]). The impact of HCMV in changing numerous immune parameters has been shown conclusively in a twin study, where identical twins varied in their HCMV infection status. It was shown that the HCMV seropositive twins had increased T cell effector memory populations and alterations in serum proteins [8].

Understanding how HCMV manipulates the immune response over time during both latent carriage and periodic reactivation of the virus leading to lytic infection requires an appreciation of the virus lifecycle. It has been shown that bone marrow resident CD34+ progenitor cells and CD14+ monocytes derived from these progenitors are sites of HCMV latent viral carriage in vivo [9]. Recent transcriptomic and single cell studies have shown that latent infection is more dynamic than previously thought with a number of different transcriptional profiles of HCMV gene expression [10,11], however HCMV latent infection of CD34+ and CD14+ cells can still be characterised by the lack of infectious virion production. Previous studies have identified particular viral genes which are transcribed during latency and are functionally important for maintaining the latent infection, including UL138 [12,13], LUNA (latent undefined nuclear antigen; UL81-82as) [14-16], US28 [17,18], UL111A (vIL-10) [19,20]. CD34+ cells latently infected in vitro with HCMV have an altered secretome which includes increased expression of chemokines that can attract CD4+ T cells as well as immune suppressive cytokines IL-10 and TGF-β [21]. In addition, it has also been shown that CD4+ T cells specific to these HCMV proteins expressed during latency can secrete IL-10 as well as having anti-viral effector functions [22,23]. Taken together this suggests that latent HCMV infection manipulates the immune response towards a more suppressive phenotype, which is in contrast to the predominantly anti-viral effector phenotype of CD4+ T cells specific to HCMV proteins expressed during lytic infection such as pp65,IE and gB [24].

It is important therefore to consider the impact of long-term carriage of HCMV, in some cases for many decades, on the immune response of the healthy host.

Does Memory Inflation of CMV specific T cell responses occur in humans?

Memory Inflation is a phenomenon associated with cytomegalovirus infection, it has been extensively studied in the murine model of cytomegalovirus (MCMV) infection. The expansion of IE1 specific CD8+ T cells in MCMV infection was originally described in the lungs of latently infected mice [25]. This work also demonstrated that T cells specific for other MCMV proteins were non-inflationary (m04, M83 and M84). In addition the inflationary CD8 T cells had an effector memory phenotype and retained the ability to make IFN-γ upon restimulation. Subsequently another group described a similar observation using the term "memory inflation", and this was observed in multiple organs and appeared to be driven by continuous activation of the T cells [26,27]. The process of "memory inflation" has subsequently been observed by other laboratories in additional mouse strains, and a number of MCMV proteins have been identified as driving "inflationary" responses, including m38, m139 and IE3 [28-31], m164 [32,33] and IE1/pp89 [26,25,34,33,35]. The term of "memory inflation" was not precisely defined in the original work using this term [26] and recently Paul Klenerman has published an updated definition with three parts *'(i) Restricted contraction following priming, leading to long-term maintained memory pool. (ii) A dominant and sustained "effector-memory" phenotype. (iii) A sustained effector functionality without features of immune exhaustion'* [36].

A characteristic of cytomegalovirus infection in both mice and men is the expansion of high avidity T cells clonotypes specific to the virus, in mice these populations are preferentially selected to "inflate" [37]. The contraction of the T cell Receptor (TCR) repertoire to focus on specific immunodominant HCMV protein epitopes has been observed in numerous studies over the last two decades [38], and the presence of these high affinity clones in HCMV responses, directed towards particular epitopes from the tegument protein pp65 [39-41], has been used as evidence that memory inflation occurs in the human host as well as the mouse [37,42]. The focusing of HCMV specific CD8+ T cells on particular TCR sequences, generating high affinity and avidity clones is seen against the IE1 protein as well as pp65 [43,44]. However, none of these studies show accumulation of these high affinity clones within the CD8+ T cell compartment over time. Where longitudinal studies have been undertaken, the frequency of these clones has been shown to be relatively stable over a number of years [44], once the primary response has resolved (based on absolute numbers of CD8+ T cell specific clones) [41]. Inflation of the frequency of HCMV specific T cells has been implied from a number of cross-sectional studies where increasing age of the subjects has been taken as a surrogate marker of time of HCMV carriage [45-49]. These cross-sectional studies also demonstrated that many of these T cells remained polyfunctional despite having a highly differentiated phenotype [39,44,50,51,46,52-54]. In addition it has been shown that the total CD8+ T cell pool (based on absolute numbers) expands to accommodate these CMV specific expansions [55,52]. Other studies have demonstrated a decline in the absolute numbers of

naïve T cells in older age irrespective of CMV sero-status [23,56]. These changes in absolute numbers of the T cell compartment may account for the observations of increased frequency of memory T cell populations in older CMV sero-positive donors [45-49] based on frequency of responses only.

The term inflation has been used in many human studies of CMV specific T cell responses to refer to large percentages (generally greater than 1.5%) of tetramer or cytokine positive T cells responding to particular HCMV peptide epitopes [57]. Certainly, there are a number of studies showing that in some donor cohorts, there are large proportions of memory T cell responses focussed on CMV in older donors, who have likely been infected for many years compared to younger donors [45,58-60,46,48,47,49]. A limited number of studies have related this increase in magnitude of CMV specific memory T cell responses to an increase in the absolute numbers of antigen specific T cells present in donors [54,61,62]. The evidence from these studies has been used by some to support the hypothesis that an increase in the size of the CMV memory T cell population over time in humans is indicative of memory inflation. However, other studies have shown that large expansions in the frequency of CMV specific memory T cells is a feature of being CMV positive irrespective of age, including studies of children as well as younger adults [63,51,64-67], this is also true when observing the absolute size of the memory T cell compartment [23,52].

Previously, we wished to investigate whether T cell responses to HCMV proteins expanded as a result of long-term carriage of the virus [23]. To address this question we have also used a cross-sectional age donor cohort to investigate whether memory CD4+ and CD8+ T cell responses expand as a result of long-term viral carriage. We used pools of peptides spanning six HCMV proteins that are expressed in lytic infection (lytic antigens), that have been shown to stimulate responses in previous studies [68,24,69,70]. We also included pools of peptides spanning HCMV proteins UL138, LUNA, vIL-10 and US28, which have been shown to be expressed by latently infected cells (latent antigens). In the paper we presented the cumulative IFN-γ responses to these lytic and latent antigens, the results showed that there was not an increase in either the breadth or magnitude of these T cell responses with increasing age. Looking at the frequency of T cell responses to 4 immunodominant HCMV proteins (pp65, gB, IE1 and IE2) in relation to the age of the donors, clearly shows that both young and old donors can have large expansions of both CD4+ and CD8+ T cell memory responses to these lytic proteins (Figure 1).

The main drawback with using cross-sectional age studies to try and identify inflationary memory responses in human subjects is the many unknowns in disease aetiology of the donors compared to murine studies. For instance in humans the time of primary infection and the initial infectious dose are not known. Also there is wide genetic variability in human subjects compared to inbred

mouse strains such as C57BL/6 or BALB/c, commonly used in MCMV memory inflation studies [28,30,71-73,26,34,37,32].

Longitudinal studies of individual donors where multiple samples over time have been examined, would clearly be a better approach for observing whether HCMV specific memory inflation occurs in humans, albeit technically challenging. There have been a limited number of studies where longitudinal samples have been included, the results have provided evidence both for and against memory T cell inflation. In an earlier study we investigated CD8+ T cell IFN-γ responses to HCMV in a small donor cohort where the frequency of T cells specific to 10 different HCMV proteins was measured in the same individuals over a period of 3 years [68]. In this study we did not observe inflation of the CD8+ T cell functional responses, indeed despite fluctuation in the magnitude of the response at the different time points, the responses were mostly stable. Fluctuations in the magnitude of HCMV responses have also been observed in another study over a period of 6 months, however this is a short period of time and there was no net increase in T cell frequency [74]; fluctuations in the magnitude of memory responses over time is also observed in murine models [32]. An Italian cohort of 25 older donors, examined whether the magnitude (based on absolute counts of peripheral blood) of pp65 and IE1 specific IFN-γ T cell responses changed over 5 years, observing a mixture of results. There was a significant increase in the magnitude of the CD8+ T cell pp65 response, but the IE specific response remained mostly stable. There were no significant changes in the CD4+ T cell pp65 and IE1 responses although there was an increase in the mean responses at the later time point [75]. Investigation of a CD4+ T cell HLA-DR7 restricted gB epitope in 2 HIV positive donors over 12 years, revealed stable responses to this epitope over time. However, the frequency of the epitope specific CD4+ T cells at the start was 16% and 18%, and as such already a highly expanded memory CD4+ T cell population [57]. Investigation of the prevalence of a γδ T cell subtype, $V\delta2^{neg}$, in CMV infection showed an “inflation” in this population in a cross-sectional age study but a longitudinal study measuring the absolute size of the $V\delta2^{neg}$ population of certain donors did not show any increases over time [76]. A recent study investigating CD8+ T cell HLA-C restricted responses does give evidence supportive of memory inflation, HLA-Cw0702 epitopes from the UL28 and IE1 proteins dominated the CD8+ T cell pool in older donors (averaging 37% of total CD8+ T cells), and in longitudinal samples from some donors there does appear to be an increase in the frequency of this response to immediate early expressed proteins [77].

Within healthy adult populations, from whom many of these studies draw their participants, the unknown factor is when an individual was infected by HCMV. It is possible that the period when memory inflation occurs post infection may be missed, as within the mouse model, inflationary responses are seen within one year of infection [26]. Therefore, it is worth investigating whether memory inflation is observable in primary HCMV infection. Due to the generally asymptomatic

nature of HCMV infection in the majority of healthy individuals, finding and following primary natural HCMV infection is not straightforward. One approach has been the use of a HCMV sero-positive donor kidney being transplanted into a HCMV sero-negative recipient and the subsequent viral infection, replication and primary immune responses are then followed in the recipient. Using this model, it has been possible to elucidate the dynamics of both CD4+ and CD8+ T cell responses to HCMV and many of these patients have been followed for up to 5 years post-transplant, as such this covers a similar time period as the murine studies of memory inflation. Key findings from this work include the importance of CD4+ T cell responses in preventing symptomatic disease, and that effector CD8+ T cells arise later but can be directly cytotoxic towards CMV presenting cells [78]. This model has also been used to track the differentiation phenotype of CMV specific T cells, including the loss of co-stimulatory molecules CD27 and CD28 [79]. Many of the published studies using this model have tracked the magnitude of CMV specific T cells or tracked specific TCR clones (using both absolute counts and frequency measures), the results from these studies has provided no direct evidence of memory inflation of the CMV specific T cells [80,81,78,82,83], however, one should remember that these patients were undergoing varying degrees of immunosuppression. In a limited number of studies where community acquired primary HCMV infection has been identified in normally healthy individuals, it has been shown that CMV specific CD8+ T cells acquire an effector phenotype during the acute phase of the infection and the numbers of CD8+ T cells increase (likely due to lymphocytosis, characteristic of natural CMV infection [1]), however by 9 months post-infection these effects are mostly resolved, although an effector memory phenotype persists [84]. In earlier studies looking at the clonality of the CMV specific CD8+ T cell response in primary infection pp65 specific responses mostly remain stable [41,59], but the frequency of IE1 specific responses may increase up to 104 weeks post infection [59].

Due to the difficulties in studying the development of memory T cell responses to cytomegalovirus in humans, the use of surrogate markers, often translated from the mouse model to identify “inflationary” populations has been used. The fractalkine receptor, CX3CR1, has been used to classify different memory CD8+ T cell populations in both mouse and human. Expression of CX3CR1 by CD8+ T cells identifies a memory population with cytotoxic effector functions independent of other differentiation markers associated with tissue homing and other previously defined memory populations [85]. In MCMV infections CX3CR1 has been shown to be a marker expressed by intermediate peripheral memory CD8+ T cells, which are generally “inflationary” populations. A similar population of memory CD8+ T cells expressing CX3CR1 has been identified in HCMV infected humans and this population was observed in response to an adenovirus based vaccine trial, suggesting this chemokine receptor could be used to identify “memory inflation” populations in humans [86]. CD85j (LIR1) expression on CD8+ T cells has been postulated to

identify a memory inflation population in HCMV infection [87]. The authors demonstrated that there was an increase in the total CD8+ population expressing CD85j in older donors, and that CMV sero-positivity led to a 19% increase in expression overall. In murine studies, inflationary memory CD8+ T cell populations have an Effector Memory (EM) phenotype (defined as $CD44^{hi}$, CD62L-, KLRG1+), in humans a similar EM population (defined as CCR7-, CD28-, CD27-, CD45RA-) can be identified within CMV tetramer specific CD8+ T cells [29]. Increased numbers of CMV specific EM cells results in increased numbers of CMV specific CD8+ T cells overall, this was also demonstrated in the primary kidney transplant model where the proportion of EM Tetramer+ (pp65) specific CD8+ T cells accumulates over time post-transplant [29].

In addition to measuring T cell responses to CMV in cross-sectional ageing studies, the HCMV specific humoral response has often been assessed. There is an increase in B cell numbers in CMV positive older donors [88], which could result in increased secretion of antigen specific immunoglobulins. A number of studies have shown that CMV specific IgM [89] and IgG titres [89-92] were significantly increased in older donors. In a longitudinal study of older donors CMV IgG titres increased significantly over 5 years [75] and in donors aged over 85 years and in poor health increased IgG significantly correlated with loss of cognitive function [61]. It has been previously suggested that high CMV IgG levels correlates with high CMV-DNA levels [93], therefore we analysed whether there was a relationship between IgG titres and CMV latent viral carriage in CD14+ monocytes using the data from the donor cohort published in 2017 [23]. Within our donor cohort we did not observe an increase in latent viral load with donor age [23], in contrast to a previous study which did see an increase in latent viral load in donors over 70 years old [90]. Neither did we see an increase in CMV IgG in relation to donor age (Figure 2A), in contrast to previous studies of CMV IgG titres. We further analysed the data set to see if CMV IgG levels were related to CMV Latent Viral Load by banding the CMV IgG measure into (i) low responses, (ii) medium responses and (iii) high responses; as shown (Figure 2B) the magnitude of the CMV IgG response did not relate to latent viral load carriage.

Overall, it is our view that there is not sufficient evidence to support the assertion that cytomegalovirus specific “memory inflation” occurs in humans, in contrast to the work in the murine field. This is due to the problems in conducting such long-term longitudinal studies in humans combined with the difficulty of knowing when primary HCMV infection occurred. It is clear that younger people can have large HCMV specific T cell expansions and it might be that the main part of the inflationary stage has already occurred at the point that these individuals have been first studied. It is interesting to note that the strongest evidence for “memory inflation” in humans, has come from longitudinal studies measuring CD8+ T cell responses to immediate early HCMV proteins as first noted in the murine studies [77,59].

Is there evidence of memory inflation of CD4+ T cells secreting IL-10 in response to HCMV?

Within the mouse model of "memory inflation" the focus has mainly been on CD8+ T cell responses, less work has focussed on the CD4+ T cell compartment. There have been limited reports of memory CD4+ T cell inflation in MCMV, with evidence that CD4+ T cell responses directed to the m09 protein arise at later time points post infection and are slightly inflationary [94]. In the murine model CD4+ T cells are important in controlling persistent MCMV infection in the salivary gland, by killing infected cells [95]. The salivary gland environment in MCMV infection has also been shown to contain IL-10 secreted by CD4+ T cells which prevents MCMV clearance [96,97]. The generation of a suppressive environment by CMV may be one method that the virus uses to modulate its environment to persist in myeloid lineage cells [4], certainly we have shown that latent infection of CD34+ cells results in the production of cellular IL-10 alongside the HCMV homolog vIL-10 (UL111a) and that these secretomes inhibit T cell effector function [21]. HCMV specific cells that secrete IL-10 or have a regulatory phenotype have been identified by ourselves and other research groups [22,98-100], however whether these T cells are inflated in CMV infection has not been definitively investigated.

Following our previous discovery of CMV specific CD4+ T cells secreting IL-10 in response to two proteins associated with latent infection, UL138 and LUNA [22], and the evidence that there are suppressive and T regulatory cells present in CMV infection, we hypothesised that lifelong carriage of HCMV infection could create an environment where IL-10 secretion was increased by skewing the CD4+ T cell viral response towards a more suppressive phenotype. Furthermore, modulation of the immune response to cytomegalovirus in old age, could explain the observation of CMV-DNA present in urine and blood of older donors [92,101]. We measured CD4+ T cell IL-10 responses to a range of HCMV proteins in a large donor cohort [23] as described in the previous section. When looking at the responses to the individual proteins which generated the most frequent IL-10 responses amongst the donor cohort, UL138, LUNA, US28, vIL-10, US3 and pp71 (Figure 3), we do not observe either inflation or deflation of these responses, similar to those observed to the cumulative results presented in [23].

An important consideration when looking for parallels between MCMV and HCMV studies, is the compartment where responses are being studied. Human studies are mainly limited to looking at immune responses present in the peripheral blood compartment at one moment in time, however most of the memory inflation populations have been identified in multiple tissue sites of the mouse including the lung [37,34,25,33] , spleen [28,31] and lymph nodes amongst many sites [26,72,94]. Murine work on IL-10 responses has also demonstrated that the salivary gland is an important site for observing these CMV specific CD4+ T cells [96,97]. It may be that the studies in humans trying to identify IL-10 CMV specific responses in peripheral blood have not necessarily been using

the correct compartment, although clearly any cross-sectional investigation of HCMV specific T cell frequencies in tissue sites is technically difficult let alone longitudinal studies which are probably impossible.

Are there Tissue Resident HCMV specific T cell responses?

Interrogating tissue resident immune responses in humans is clearly technically challenging, with most studies focus being on the peripheral blood compartment because it is easy to access and not detrimental to the health of the donor. Other tissue sites can be accessed during medical procedures and investigations, however this may mean that the donor is unwell, although depending on individual symptoms the samples obtained may still be useable. CMV and CMV specific T cell responses have been investigated in samples acquired via other medical procedures, including intestinal IL-10 CD4+ T cells specific for HCMV [96], alveolar macrophages from Bronchoalveolar Lavage have been shown to be sites of HCMV reactivation *in vivo* in the lung [102]. Secondary lymphoid organs including, Lymph nodes, spleen, bone marrow and tonsils are often removed for a variety of medical reasons, and these tissue sites have also been examined for anti-viral T cell responses [103-106]. Cadavers have been used to access multiple tissue sites from the same donor and recently CD8+ T cell responses, specific for pp65 and IE proteins, have been examined in 24 CMV sero-positive donors at multiple tissue sites including blood, Bone Marrow, Spleen, multiple Lymph Nodes and mucosal sites including the lungs and intestines. CMV specific CD8+ T cells were identified at high frequency at several tissue sites including the lungs, blood, bone marrow and lymph nodes [107]. CD8+ T cell responses specific to pp65 and IE1 have also been observed in paired blood and lymph node samples from living donors, examination of clonal responses has shown that during CMV reactivation new clones generated in the peripheral blood do not arise from the lymph node [104], the CD8+ CMV specific T cells present in the lymph node are generally polyfunctional memory cells, with only low expression of CX3CR1 [103]. CMV specific CD8+ T cell responses have also been investigated in bone marrow specimens, this study showed an increase in Effector Memory CD8+ T cells in bone marrow samples, although the frequency of CMV specific T cells was lower in the bone marrow compared to peripheral blood in this donor cohort [106].

Most studies of HCMV specific T cells so far in tissue sites have neglected CD4+ T cell responses and functional responses, relying on phenotyping the total population and specificity by tetramers containing pp65 and IE1 derived peptides in order to quantify the antigen specific cells. However, it is clear that there are HCMV CD4+ and CD8+ T cell responses to a much broader range of proteins than these two immunodominant proteins [70,69,50,68,24,23,22]. We have conducted preliminary studies looking at CD4+ and CD8+ T cell responses isolated from paired peripheral blood and bone marrow samples to 11 different HCMV proteins measuring IFNγ and IL-10

responses by Fluorospot (Figure 4). The results from the CD8+ T cell compartment show that IFNγ responses to all 11 proteins are detectable in the peripheral blood, but there are no detectable CD8+ T cell responses to UL138 and LUNA in the bone marrow specimen. When looking at CD4+ T cell responses both IFNγ and IL-10 responses show a distinct profile in the bone marrow cells compared to peripheral blood, for instance there is an IL-10 response to IE1 in the bone marrow sample, we have rarely observed IL-10 responses to this protein in peripheral blood [23]. Also, the IFNγ CD4+ T cell response to the latency associated proteins is at a higher frequency in the bone marrow sample compared to the peripheral blood sample. This initial result suggests that it will be important to look for both IFNγ and IL-10 T cell responses to HCMV in sites other than peripheral blood.

The importance of understanding anti-viral functional responses in HCMV.

The studies into HCMV immune responses discussed previously have relied on identification of responses by tetramer flow cytometry, peptide or viral lysate stimulation in ELISPOT or intracellular cytokine assays, as such this studies antigen stimulation in the absence of intact functional virus expressing immune evasion molecules [5]. This contrasts with the numerous murine studies, where the “memory inflation” phenomenon can be interrogated by using viral strains with inflationary or immune evasion proteins mutated and in mice which have also been genetically manipulated to remove specific immune cell populations or irradiated and specific T cell populations adoptively transferred. In humans the immune responses we observe are a result of infection with a “wild type” virus and the effects of viral immune evasion, manipulation and modulation of the anti-cytomegalovirus immune response most likely enables the virus to persist and establish latent infections [4]. However we are not able to manipulate either the viral encoded factors or the host immune response *in vivo* as is possible in murine models. To improve our understanding of the generation, maintenance and functionality of HCMV specific T cell responses requires development of appropriate *in vitro* experimental models, where gene editing approaches and analysis of isolated immune cell populations can be performed.

We have developed a viral dissemination assay utilising primary dermal fibroblasts from individual donors, grown from a 2mm punch biopsy (Figure 5A), which allows the measurement of CD8+ T cell anti-viral functionality in a fully autologous system (Figure 5B) [68], and we have modified the assay to allow the interrogation of NK cells [108] and both CD4+ and CD8+ T cells anti-viral responses against lytic infection in myeloid cells [24], furthermore the myeloid cell based assay will also allow the interrogation of immune cell interactions with experimental latent infection in monocytes (Figure 5C).

The viral dissemination assay has shown that pp65, IE1, pp71 and US3 specific CD8+ T cells are able to control the spread of the virus, this direct anti-viral control is present in CMV specific CD8+ T cells isolated directly *ex vivo* from peripheral blood [68]. Using a version of this assay (Figure 5B), the role of NK cells expressing the inhibitory receptor LIR1 to control viral spread was elucidated [108]. Ongoing investigations have shown that CMV specific CD8+ T cell responses in isolation are effective in preventing the spread of the virus encoding a full complement of immune evasion molecules, by keeping the virus static in cells, but removal of CD8+ T cells from the dissemination assay allows the virus to restart spreading through the dermal fibroblast monolayer. However when using a mutant cytomegalovirus with US2-11 genes deleted in the viral dissemination assay, this HCMV gene region encodes immune evasion molecules that interfere with antigen processing and presentation pathways [5], *ex vivo* CD8+ T cells alone are virucidal (data not shown). Co-culture of whole PBMC with clinical strains of the virus (expressing all immune evasion genes) was virucidal. We hypothesise that the interaction between the different immune cell subsets (CD4/8+ T cell, NK cells and Monocytes) augments the cell mediated response overcoming virus mediated immune evasion and leading to killing of virus infected cells. The mechanism of this virucidal activity is currently being investigated.

Using the modified viral dissemination assay (Figure 5C), we have shown that CD4+ T cells isolated from CMV sero-positive donors are able to control viral dissemination at very low effector to target (E:T) ratios and that CD4+ T cells from CMV sero-negative donors cannot [24]. In this study we had one donor aged over 70 years, and at the lowest E:T ratios their CD4+ T cells were not controlling viral dissemination as efficiently as the 4 younger donors analysed, suggesting that there may be a slight loss of quality in the functional control of viral dissemination in older CMV sero-positive donors. The loss of quality of the anti-cytomegalovirus immune response in older donors may explain why reactivating virus is detected in urine from older and not younger donors [92]. Utilising both the fibroblast and myeloid cell based viral dissemination experimental models will enable the interrogation of whether tissue resident anti-HCMV T cells are more effective at controlling viral dissemination than T cells isolated from peripheral blood, and whether different T cell memory populations (e.g. effector memory or CX3CR1+ memory) have differing abilities to control the spread of or kill HCMV infected cells.

Conclusion

Investigation of T cell responses to HCMV are challenging but appropriate study design such as conducting more longitudinal studies in a wider range of donor cohorts will help to elucidate whether “memory inflation” is a phenomenon that occurs. Also these studies will enable a greater understanding of how HCMV specific T cell memory evolves over time in the human host. Furthermore increasing the breadth of cytomegalovirus protein responses analysed including using

intact wildtype virus in assays that measure anti-viral effector function will more closely replicate the work performed in the MCMV model system. Performing analyses of T cell responses from a wider range of tissue sites and utilising a range of functional outputs in the face of viral immune evasion strategies will increase our understanding of the maintenance of the immune response to HCMV in both lytic and latent infection. Such a fundamental understanding of the immunobiology of HCMV should inform strategies to better target the virus and possibility of removing latent virus infected cells to reduce complications of virus reactivation in the immunosuppressed transplant setting.

REFERENCES

1. Gandhi MK, Khanna R (2004) Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. Lancet Infect Dis 4 (12):725-738. doi:10.1016/S1473-3099(04)01202-2

2. Crough T, Khanna R (2009) Immunobiology of human cytomegalovirus: from bench to bedside. Clin Microbiol Rev 22 (1):76-98. doi:10.1128/CMR.00034-08

3. Jackson SE, Mason GM, Wills MR (2011) Human cytomegalovirus immunity and immune evasion. Virus Res 157 (2):151-160. doi:10.1016/j.virusres.2010.10.031

4. Wills MR, Poole E, Lau B, Krishna B, Sinclair JH (2015) The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies? Cell Mol Immunol 12 (2):128-138. doi:10.1038/cmi.2014.75

5. Jackson SE, Redeker A, Arens R, van Baarle D, van den Berg SPH, Benedict CA, Cicin-Sain L, Hill AB, Wills MR (2017) CMV immune evasion and manipulation of the immune system with aging. Geroscience 39 (3):273-291. doi:10.1007/s11357-017-9986-6

6. Sinclair J, Sissons P (2006) Latency and reactivation of human cytomegalovirus. J Gen Virol 87 (Pt 7):1763-1779. doi:10.1099/vir.0.81891-0

7. Picarda G, Benedict CA (2018) Cytomegalovirus: Shape-Shifting the Immune System. J Immunol 200 (12):3881-3889. doi:10.4049/jimmunol.1800171

8. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJ, Furman D, Shen-Orr S, Dekker CL, Swan GE, Butte AJ, Maecker HT, Davis MM (2015) Variation in the human immune system is largely driven by non-heritable influences. Cell 160 (1-2):37-47. doi:10.1016/j.cell.2014.12.020

9. Poole E, Sinclair J (2015) Sleepless latency of human cytomegalovirus. Med Microbiol Immunol 204 (3):421-429. doi:10.1007/s00430-015-0401-6

10. Cheng S, Caviness K, Buehler J, Smithey M, Nikolich-Zugich J, Goodrum F (2017) Transcriptome-wide characterization of human cytomegalovirus in natural infection and experimental latency. Proc Natl Acad Sci U S A 114 (49):E10586-E10595. doi:10.1073/pnas.1710522114

11. Shnayder M, Nachshon A, Krishna B, Poole E, Boshkov A, Binyamin A, Maza I, Sinclair J, Schwartz M, Stern-Ginossar N (2018) Defining the Transcriptional Landscape during Cytomegalovirus Latency with Single-Cell RNA Sequencing. MBio 9 (2):18. doi:10.1128/mBio.00013-18

12. Goodrum F, Reeves M, Sinclair J, High K, Shenk T (2007) Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro. Blood 110 (3):937-945. doi:10.1182/blood-2007-01-070078

13. Lee SH, Caviness K, Albright ER, Lee JH, Gelbmann CB, Rak M, Goodrum F, Kalejta RF (2016) Long and Short Isoforms of the Human Cytomegalovirus UL138 Protein Silence IE Transcription and Promote Latency. J Virol 90 (20):9483-9494. doi:10.1128/JVI.01547-16

14. Bego M, Maciejewski J, Khaiboullina S, Pari G, St Jeor S (2005) Characterization of an antisense transcript spanning the UL81-82 locus of human cytomegalovirus. J Virol 79 (17):11022-11034. doi:10.1128/JVI.79.17.11022-11034.2005

15. Reeves MB, Sinclair JH (2010) Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter. J Gen Virol 91 (Pt 3):599-604. doi:10.1099/vir.0.015602-0

16. Poole EL, Kew VG, Lau JCH, Murray MJ, Stamminger T, Sinclair JH, Reeves MB (2018) A Virally Encoded DeSUMOylase Activity Is Required for Cytomegalovirus Reactivation from Latency. Cell Rep 24 (3):594-606. doi:10.1016/j.celrep.2018.06.048

17. Beisser PS, Laurent L, Virelizier JL, Michelson S (2001) Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. J Virol 75 (13):5949-5957. doi:10.1128/JVI.75.13.5949-5957.2001

18. Krishna BA, Poole EL, Jackson SE, Smit MJ, Wills MR, Sinclair JH (2017) Latency-Associated Expression of Human Cytomegalovirus US28 Attenuates Cell Signaling Pathways To Maintain Latent Infection. MBio 8 (6):17. doi:10.1128/mBio.01754-17

19. Jenkins C, Abendroth A, Slobedman B (2004) A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection. J Virol 78 (3):1440-1447. doi:10.1128/Jvi.78.3.1440-1447.2004

20. Poole E, Avdic S, Hodkinson J, Jackson SE, Wills M, Slobedman B, Sinclair J (2014) Latency-associated viral interleukin-10 (IL-10) encoded by human cytomegalovirus modulates cellular IL-10 and CCL8 Secretion during latent infection through changes in the cellular microRNA hsa-miR-92a. J Virol 88 (24):13947-13955. doi:10.1128/JVI.02424-14

21. Mason GM, Poole E, Sissons JG, Wills MR, Sinclair JH (2012) Human cytomegalovirus latency alters the cellular secretome, inducing cluster of differentiation (CD)4+ T-cell migration and suppression of effector function. Proc Natl Acad Sci U S A 109 (36):14538-14543. doi:10.1073/pnas.1204836109

22. Mason GM, Jackson SE, Okecha G, Poole E, Sissons JG, Sinclair J, Wills MR (2013) Human cytomegalovirus latency-associated proteins elicit immune-suppressive IL-10 producing CD4(+) T cells. PLoS Pathog 9 (10):e1003635. doi:10.1371/journal.ppat.1003635

23. Jackson SE, Sedikides GX, Okecha G, Poole EL, Sinclair JH, Wills MR (2017) Latent Cytomegalovirus (CMV) Infection Does Not Detrimentally Alter T Cell Responses in the Healthy Old, But Increased Latent CMV Carriage Is Related to Expanded CMV-Specific T Cells. Front Immunol 8:733. doi:10.3389/fimmu.2017.00733

24. Jackson SE, Sedikides GX, Mason GM, Okecha G, Wills MR (2017) Human Cytomegalovirus (HCMV)-Specific CD4+ T Cells Are Polyfunctional and Can Respond to HCMV-Infected Dendritic Cells In Vitro. J Virol 91 (6):16. doi:10.1128/JVI.02128-16

25. Holtappels R, Pahl-Seibert MF, Thomas D, Reddehase MJ (2000) Enrichment of Immediate-Early 1 (m123/pp89) Peptide-Specific CD8 T Cells in a Pulmonary CD62Llo Memory-Effector Cell Pool during Latent Murine Cytomegalovirus Infection of the Lungs. J Virol 74 (24):11495-11503. doi:10.1128/jvi.74.24.11495-11503.2000

26. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, Phillips RE, Klenerman P (2003) Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. J Immunol 170 (4):2022-2029

27. Karrer U, Sierro S, Wagner M, Oxenius A, Hengel H, Koszinowski UH, Phillips RE, Klenerman P (2003) Memory inflation: Continous accumulation of antiviral CD8(+) T cells over time. (vol 170,

pg 2022, 2003). Journal of Immunology 171 (7):3895-3895. doi:DOI 10.4049/jimmunol.171.7.3895-b

28. Snyder CM, Cho KS, Bonnett EL, van Dommelen S, Shellam GR, Hill AB (2008) Memory Inflation during Chronic Viral Infection Is Maintained by Continuous Production of Short-Lived, Functional T Cells. Immunity 29 (4):650-659. doi:10.1016/j.immuni.2008.07.017

29. Redeker A, Remmerswaal EBM, van der Gracht ETI, Welten SPM, Höllt T, Koning F, Cicin-Sain L, Nikolich-Žugich J, ten Berge IJM, van Lier RAW, van Unen V, Arens R (2018) The Contribution of Cytomegalovirus Infection to Immune Senescence Is Set by the Infectious Dose. Front Immunol 8:1953. doi:10.3389/fimmu.2017.01953

30. Turula H, Smith CJ, Grey F, Zurbach KA, Snyder CM (2013) Competition between T cells maintains clonal dominance during memory inflation induced by MCMV. Eur J Immunol 43 (5):1252-1263. doi:10.1002/eji.201242940

31. Munks MW, Cho KS, Pinto AK, Sierro S, Klenerman P, Hill AB (2006) Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. J Immunol 177 (1):450-458

32. Holtappels R, Lemmermann NA, Podlech J, Ebert S, Reddehase MJ (2016) Reconstitution of CD8 T Cells Protective against Cytomegalovirus in a Mouse Model of Hematopoietic Cell Transplantation: Dynamics and Inessentiality of Epitope Immunodominance. Front Immunol 7:232. doi:10.3389/fimmu.2016.00232

33. Holtappels R, Thomas D, Podlech J, Reddehase MJ (2002) Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2d haplotype. J Virol 76 (1):151-164

34. Trgovcich J, Kincaid M, Thomas A, Griessl M, Zimmerman P, Dwivedi V, Bergdall V, Klenerman P, Cook CH (2016) Cytomegalovirus Reinfections Stimulate CD8 T-Memory Inflation. PLoS One 11 (11):e0167097. doi:10.1371/journal.pone.0167097

35. Karrer U, Wagner M, Sierro S, Oxenius A, Hengel H, Dumrese T, Freigang S, Koszinowski UH, Phillips RE, Klenerman P (2004) Expansion of protective CD8+ T-cell responses driven by recombinant cytomegaloviruses. J Virol 78 (5):2255-2264

36. Klenerman P (2018) The (gradual) rise of memory inflation. Immunol Rev 283 (1):99-112. doi:10.1111/imr.12653

37. Morabito KM, Ruckwardt TJ, Bar-Haim E, Nair D, Moin SM, Redwood AJ, Price DA, Graham BS (2018) Memory Inflation Drives Tissue-Resident Memory CD8(+) T Cell Maintenance in the Lung After Intranasal Vaccination With Murine Cytomegalovirus. Front Immunol 9:1861. doi:10.3389/fimmu.2018.01861

38. Schober K, Buchholz VR, Busch DH (2018) TCR repertoire evolution during maintenance of CMV-specific T-cell populations. Immunol Rev 283 (1):113-128. doi:10.1111/imr.12654

39. Price DA, Brenchley JM, Ruff LE, Betts MR, Hill BJ, Roederer M, Koup RA, Migueles SA, Gostick E, Wooldridge L, Sewell AK, Connors M, Douek DC (2005) Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. J Exp Med 202 (10):1349-1361. doi:10.1084/jem.20051357

40. Trautmann L, Rimbert M, Echasserieau K, Saulquin X, Neveu B, Dechanet J, Cerundolo V, Bonneville M (2005) Selection of T cell clones expressing high-affinity public TCRs within human cytomegalovirus-specific CD8 T cell responses. J Immunol 175 (9):6123-6132. doi:DOI 10.4049/jimmunol.175.9.6123

41. Day EK, Carmichael AJ, Ten Berge IJM, Waller ECP, Sissons JGP, Wills MR (2007) Rapid CD8(+) T cell repertoire focusing and selection of high-affinity clones into memory following primary infection with a persistent human virus: Human Cytomegalovirus. J Immunol 179 (5):3203-3213

42. Luo XH, Meng Q, Rao M, Liu Z, Paraschoudi G, Dodoo E, Maeurer M (2018) The impact of inflationary cytomegalovirus-specific memory T cells on anti-tumour immune responses in patients with cancer. Immunology 155 (3):294-308. doi:10.1111/imm.12991

43. Khan N, Cobbold M, Keenan R, Moss PAH (2002) Comparative analysis of CD8(+) T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. J Infect Dis 185 (8):1025-1034. doi:Doi 10.1086/339963

44. Iancu EM, Corthesy P, Baumgaertner P, Devevre E, Voelter V, Romero P, Speiser DE, Rufer N (2009) Clonotype Selection and Composition of Human CD8 T Cells Specific for Persistent Herpes Viruses Varies with Differentiation but Is Stable Over Time. J Immunol 183 (1):319-331. doi:10.4049/jimmunol.0803647

45. Khan N, Shariff N, Cobbold M, Bruton R, Ainsworth JA, Sinclair AJ, Nayak L, Moss PA (2002) Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol 169 (4):1984-1992

46. Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Wurzner R, Schonitzer D, Grubeck-Loebenstein B (2005) Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8(+) T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. J Virol 79 (6):3675-3683. doi:10.1128/Jvi.79.6.3675-3683.2005

47. Khan N, Hislop A, Gudgeon N, Cobbold M, Khanna R, Nayak L, Rickinson AB, Moss PA (2004) Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. J Immunol 173 (12):7481-7489

48. Pita-Lopez ML, Gayoso I, DelaRosa O, Casado JG, Alonso C, Munoz-Gomariz E, Tarazona R, Solana R (2009) Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. Immun Ageing 6 (1):11. doi:10.1186/1742-4933-6-11

49. Schwanninger A, Weinberger B, Weiskopf D, Herndler-Brandstetter D, Reitinger S, Gassner C, Schennach H, Parson W, Wurzner R, Grubeck-Loebenstein B (2008) Age-related appearance of a CMV-specific high-avidity CD8+ T cell clonotype which does not occur in young adults. Immun Ageing 5 (1):14. doi:10.1186/1742-4933-5-14

50. Bajwa M, Vita S, Vescovini R, Larsen M, Sansoni P, Terrazzini N, Caserta S, Thomas D, Davies KA, Smith H, Kern F (2017) CMV-Specific T-cell Responses at Older Ages: Broad Responses With a Large Central Memory Component May Be Key to Long-term Survival. J Infect Dis 215 (8):1212-1220. doi:10.1093/infdis/jix080

51. Lelic A, Verschoor CP, Ventresca M, Parsons R, Evelegh C, Bowdish D, Betts MR, Loeb MB, Bramson JL (2012) The polyfunctionality of human memory CD8+ T cells elicited by acute and chronic virus infections is not influenced by age. PLoS Pathog 8 (12):e1003076. doi:10.1371/journal.ppat.1003076

52. Lachmann R, Bajwa M, Vita S, Smith H, Cheek E, Akbar A, Kern F (2012) Polyfunctional T cells accumulate in large human cytomegalovirus-specific T cell responses. J Virol 86 (2):1001-1009. doi:10.1128/JVI.00873-11

53. Vescovini R, Fagnoni FF, Telera AR, Bucci L, Pedrazzoni M, Magalini F, Stella A, Pasin F, Medici MC, Calderaro A, Volpi R, Monti D, Franceschi C, Nikolich-Zugich J, Sansoni P (2014) Naive and memory CD8 T cell pool homeostasis in advanced aging: impact of age and of antigen-specific responses to cytomegalovirus. Age (Dordr) 36 (2):625-640. doi:10.1007/s11357-013-9594-z

54. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, Pedrazzoni M, Bucci L, Monti D, Medici MC, Chezzi C, Franceschi C, Sansoni P (2007) Massive load of functional effector CD4(+) and CD8(+) T cells against cytomegalovirus in very old subjects. J Immunol 179 (6):4283-4291

55. Lindau P, Mukherjee R, Gutschow MV, Vignali M, Warren EH, Riddell SR, Makar KW, Turtle CJ, Robins HS (2019) Cytomegalovirus Exposure in the Elderly Does Not Reduce CD8 T Cell Repertoire Diversity. J Immunol 202 (2):476-483. doi:10.4049/jimmunol.1800217

56. Wertheimer AM, Bennett MS, Park B, Uhrlaub JL, Martinez C, Pulko V, Currier NL, Nikolich-Zugich D, Kaye J, Nikolich-Zugich J (2014) Aging and cytomegalovirus infection differentially and jointly affect distinct circulating T cell subsets in humans. J Immunol 192 (5):2143-2155. doi:10.4049/jimmunol.1301721

57. Abana CO, Pilkinton MA, Gaudieri S, Chopra A, McDonnell WJ, Wanjalla C, Barnett L, Gangula R, Hager C, Jung DK, Engelhardt BG, Jagasia MH, Klenerman P, Phillips EJ, Koelle DM, Kalams SA, Mallal SA (2017) Cytomegalovirus (CMV) Epitope-Specific CD4(+) T Cells Are Inflated in HIV(+) CMV(+) Subjects. J Immunol 199 (9):3187-3201. doi:10.4049/jimmunol.1700851

58. Pourgheysari B, Khan N, Best D, Bruton R, Nayak L, Moss PA (2007) The cytomegalovirus-specific CD4+ T-cell response expands with age and markedly alters the CD4+ T-cell repertoire. J Virol 81 (14):7759-7765. doi:10.1128/JVI.01262-06

59. Khan N, Best D, Bruton R, Nayak L, Rickinson AB, Moss PA (2007) T cell recognition patterns of immunodominant cytomegalovirus antigens in primary and persistent infection. J Immunol 178 (7):4455-4465

60. Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, Muller CA, Pircher H, Pawelec G (2003) Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). Exp Gerontol 38 (8):911-920

61. Vescovini R, Biasini C, Telera AR, Basaglia M, Stella A, Magalini F, Bucci L, Monti D, Lazzarotto T, Dal Monte P, Pedrazzoni M, Medici MC, Chezzi C, Franceschi C, Fagnoni FF, Sansoni P (2010) Intense Antiextracellular Adaptive Immune Response to Human Cytomegalovirus in Very Old Subjects with Impaired Health and Cognitive and Functional Status. J Immunol 184 (6):3242-3249. doi:10.4049/jimmunol.0902890

62. Vescovini R, Telera A, Fagnoni FF, Biasini C, Medici MC, Valcavi P, di Pede P, Lucchini G, Zanlari L, Passeri G, Zanni F, Chezzi C, Franceschi C, Sansoni P (2004) Different contribution of EBV and CMV infections in very long-term carriers to age-related alterations of CD8(+) T cells. Exp Gerontol 39 (8):1233-1243. doi:10.1016/j.exger.2004.04.004

63. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, Kidd BA, Maecker HT, Concannon P, Dekker CL, Thomas PG, Davis MM (2015) Cytomegalovirus infection enhances the immune response to influenza. Sci Transl Med 7 (281):281ra243. doi:10.1126/scitranslmed.aaa2293

64. Weltevrede M, Eilers R, de Melker HE, van Baarle D (2016) Cytomegalovirus persistence and T-cell immunosenescence in people aged fifty and older: A systematic review. Exp Gerontol 77:87-95. doi:10.1016/j.exger.2016.02.005

65. Burton CE, Sester M, Robinson JL, Eurich DT, Preiksaitis JK, Urschel S (2018) Assigning Cytomegalovirus Status in Children Awaiting Organ Transplant: Viral Shedding, CMV-Specific T Cells, and CD27-CD28-CD4+ T Cells. J Infect Dis 218 (8):1205-1209. doi:10.1093/infdis/jiy309

66. Pera A, Vasudev A, Tan C, Kared H, Solana R, Larbi A (2017) CMV induces expansion of highly polyfunctional CD4+ T cell subset coexpressing CD57 and CD154. J Leukoc Biol 101 (2):555-566. doi:10.1189/jlb.4A0316-112R

67. Pera A, Caserta S, Albanese F, Blowers P, Morrow G, Terrazzini N, Smith HE, Rajkumar C, Reus B, Msonda JR, Verboom M, Hallensleben M, Blasczyk R, Davies KA, Kern F (2018) CD28(null) pro-atherogenic CD4 T-cells explain the link between CMV infection and an increased risk of cardiovascular death. Theranostics 8 (16):4509-4519. doi:10.7150/thno.27428

68. Jackson SE, Mason GM, Okecha G, Sissons JG, Wills MR (2014) Diverse specificities, phenotypes, and antiviral activities of cytomegalovirus-specific CD8+ T cells. J Virol 88 (18):10894-10908. doi:10.1128/JVI.01477-14

69. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleath PR, Grabstein KH, Hosken NA, Kern F, Nelson JA, Picker LJ (2005) Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med 202 (5):673-685. doi:10.1084/jem.20050882

70. Sylwester A, Nambiar KZ, Caserta S, Klenerman P, Picker LJ, Kern F (2016) A new perspective of the structural complexity of HCMV-specific T-cell responses. Mech Ageing Dev 158:14-22. doi:10.1016/j.mad.2016.03.002

71. Dekhtiarenko I, Ratts RB, Blatnik R, Lee LN, Fischer S, Borkner L, Oduro JD, Marandu TF, Hoppe S, Ruzsics Z, Sonnemann JK, Mansouri M, Meyer C, Lemmermann NA, Holtappels R, Arens R, Klenerman P, Fruh K, Reddehase MJ, Riemer AB, Cicin-Sain L (2016) Peptide Processing Is Critical for T-Cell Memory Inflation and May Be Optimized to Improve Immune Protection by CMV-Based Vaccine Vectors. PLoS Pathog 12 (12):e1006072. doi:10.1371/journal.ppat.1006072

72. Borkner L, Sitnik KM, Dekhtiarenko I, Pulm AK, Tao R, Drexler I, Cicin-Sain L (2017) Immune Protection by a Cytomegalovirus Vaccine Vector Expressing a Single Low-Avidity Epitope. J Immunol 199 (5):1737-1747. doi:10.4049/jimmunol.1602115

73. Bolinger B, Sims S, O'Hara G, de Lara C, Tchilian E, Firner S, Engeler D, Ludewig B, Klenerman P (2013) A new model for CD8+ T cell memory inflation based upon a recombinant adenoviral vector. J Immunol 190 (8):4162-4174. doi:10.4049/jimmunol.1202665

74. Crough T, Burrows JM, Fazou C, Walker S, Davenport MP, Khanna R (2005) Contemporaneous fluctuations in T cell responses to persistent herpes virus infections. Eur J Immunol 35 (1):139-149. doi:10.1002/eji.200425548

75. Vescovini R, Telera AR, Pedrazzoni M, Abbate B, Rossetti P, Verzicco I, Arcangeletti MC, Medici MC, Calderaro A, Volpi R, Sansoni P, Fagnoni FF (2016) Impact of Persistent Cytomegalovirus Infection on Dynamic Changes in Human Immune System Profile. PLoS One 11 (3):e0151965. doi:10.1371/journal.pone.0151965

76. Alejenef A, Pachnio A, Halawi M, Christmas SE, Moss PA, Khan N (2014) Cytomegalovirus drives Vdelta2neg gammadelta T cell inflation in many healthy virus carriers with increasing age. Clin Exp Immunol 176 (3):418-428. doi:10.1111/cei.12297

77. Hosie L, Pachnio A, Zuo J, Pearce H, Riddell S, Moss P (2017) Cytomegalovirus-Specific T Cells Restricted by HLA-Cw*0702 Increase Markedly with Age and Dominate the CD8+ T-Cell Repertoire in Older People. Front Immunol 8:1776. doi:10.3389/fimmu.2017.01776

78. Gamadia LE, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, Ten Berge IJ (2003) Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. Blood 101 (7):2686-2692. doi:10.1182/blood-2002-08-2502

79. van de Berg PJ, van Stijn A, Ten Berge IJ, van Lier RA (2008) A fingerprint left by cytomegalovirus infection in the human T cell compartment. J Clin Virol 41 (3):213-217. doi:10.1016/j.jcv.2007.10.016

80. Klarenbeek PL, Remmerswaal EB, ten Berge IJ, Doorenspleet ME, van Schaik BD, Esveldt RE, Koch SD, ten Brinke A, van Kampen AH, Bemelman FJ, Tak PP, Baas F, de Vries N, van Lier RA (2012) Deep sequencing of antiviral T-cell responses to HCMV and EBV in humans reveals a stable repertoire that is maintained for many years. PLoS Pathog 8 (9):e1002889. doi:10.1371/journal.ppat.1002889

81. Hertoghs KM, Moerland PD, van Stijn A, Remmerswaal EB, Yong SL, van de Berg PJ, van Ham SM, Baas F, ten Berge IJ, van Lier RA (2010) Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. J Clin Invest 120 (11):4077-4090. doi:10.1172/JCI42758

82. Gamadia LE, Rentenaar RJ, van Lier RA, ten Berge IJ (2004) Properties of CD4(+) T cells in human cytomegalovirus infection. Hum Immunol 65 (5):486-492. doi:10.1016/j.humimm.2004.02.020

689 83. Gamadia LE, van Leeuwen EMM, Remmerswaal EBM, Yong SL, Surachno S, Dillen PMEWV,
690 ten Berge IJM, van Lier RAW (2004) The size and phenotype of virus-specific T cell populations is
691 determined by repetitive antigenic stimulation and environmental cytokines. J Immunol 172
692 (10):6107-6114

693 84. Riou R, Bressollette-Bodin C, Boutoille D, Gagne K, Rodallec A, Lefebvre M, Raffi F, Senitzer
694 D, Imbert-Marcille BM, Retiere C (2017) Severe Symptomatic Primary Human Cytomegalovirus
695 Infection despite Effective Innate and Adaptive Immune Responses. J Virol 91 (5):16.
696 doi:10.1128/JVI.02245-16

697 85. Bottcher JP, Beyer M, Meissner F, Abdullah Z, Sander J, Hochst B, Eickhoff S, Rieckmann JC,
698 Russo C, Bauer T, Flecken T, Giesen D, Engel D, Jung S, Busch DH, Protzer U, Thimme R, Mann
699 M, Kurts C, Schultze JL, Kastenmuller W, Knolle PA (2015) Functional classification of memory
700 CD8(+) T cells by CX3CR1 expression. Nat Commun 6:8306. doi:10.1038/ncomms9306

701 86. Gordon CL, Lee LN, Swadling L, Hutchings C, Zinser M, Highton AJ, Capone S, Folgori A,
702 Barnes E, Klenerman P (2018) Induction and Maintenance of CX3CR1-Intermediate Peripheral
703 Memory CD8(+) T Cells by Persistent Viruses and Vaccines. Cell Rep 23 (3):768-782.
704 doi:10.1016/j.celrep.2018.03.074

705 87. Northfield J, Lucas M, Jones H, Young NT, Klenerman P (2005) Does memory improve with
706 age? CD85j (ILT-2/LIR-1) expression on CD8 T cells correlates with 'memory inflation' in human
707 cytomegalovirus infection. Immunol Cell Biol 83 (2):182-188. doi:10.1111/j.1440-
708 1711.2005.01321.x

709 88. Chidrawar S, Khan N, Wei W, McLarnon A, Smith N, Nayak L, Moss P (2009)
710 Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid
711 subsets within healthy individuals. Clin Exp Immunol 155 (3):423-432. doi:10.1111/j.1365-
712 2249.2008.03785.x

713 89. McVoy MA, Adler SP (1989) Immunologic evidence for frequent age-related cytomegalovirus
714 reactivation in seropositive immunocompetent individuals. J Infect Dis 160 (1):1-10

715 90. Parry HM, Zuo J, Frumento G, Mirajkar N, Inman C, Edwards E, Griffiths M, Pratt G, Moss P
716 (2016) Cytomegalovirus viral load within blood increases markedly in healthy people over the age
717 of 70 years. Immun Ageing 13 (1):1. doi:10.1186/s12979-015-0056-6

718 91. Alonso Arias R, Moro-Garcia MA, Echeverria A, Solano-Jaurrieta JJ, Suarez-Garcia FM,
719 Lopez-Larrea C (2013) Intensity of the humoral response to cytomegalovirus is associated with the
720 phenotypic and functional status of the immune system. J Virol 87 (8):4486-4495.
721 doi:10.1128/JVI.02425-12

722 92. Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, Glaser R (2007) Chronic
723 herpesvirus reactivation occurs in aging. Exp Gerontol 42 (6):563-570.
724 doi:10.1016/j.exger.2007.01.005

725 93. Fornara C, Furione M, Lilleri D, Cane I, Revello MG, Zavattoni M, Gerna G (2015) Primary
726 human cytomegalovirus infections: kinetics of ELISA-IgG and neutralizing antibody in
727 pauci/asymptomatic pregnant women vs symptomatic non-pregnant subjects. J Clin Virol 64:45-51.
728 doi:10.1016/j.jcv.2015.01.004

729 94. Arens R, Wang P, Sidney J, Loewendorf A, Sette A, Schoenberger SP, Peters B, Benedict CA
730 (2008) Cutting edge: murine cytomegalovirus induces a polyfunctional CD4 T cell response. J
731 Immunol 180 (10):6472-6476

732 95. Verma S, Weiskopf D, Gupta A, McDonald B, Peters B, Sette A, Benedict CA (2015)
733 Cytomegalovirus-Specific CD4 T Cells Are Cytolytic and Mediate Vaccine Protection. J Virol 90
734 (2):650-658. doi:10.1128/JVI.02123-15

735 96. Clement M, Marsden M, Stacey MA, Abdul-Karim J, Gimeno Brias S, Costa Bento D, Scurr MJ,
736 Ghazal P, Weaver CT, Carlesso G, Clare S, Jones SA, Godkin A, Jones GW, Humphreys IR
737 (2016) Cytomegalovirus-Specific IL-10-Producing CD4+ T Cells Are Governed by Type-I IFN-

738 Induced IL-27 and Promote Virus Persistence. PLoS Pathog 12 (12):e1006050.
739 doi:10.1371/journal.ppat.1006050

740 97. Jost NH, Abel S, Hutzler M, Sparwasser T, Zimmermann A, Roers A, Muller W, Klopfleisch R,
741 Hengel H, Westendorf AM, Buer J, Hansen W (2014) Regulatory T cells and T-cell-derived IL-10
742 interfere with effective anti-cytomegalovirus immune response. Immunol Cell Biol 92 (10):860-871.
743 doi:10.1038/icb.2014.62

744 98. Schwele S, Fischer AM, Brestrich G, Wlodarski MW, Wagner L, Schmueck M, Roemhild A,
745 Thomas S, Hammer MH, Babel N, Kurtz A, Maciejewski JP, Reinke P, Volk HD (2012)
746 Cytomegalovirus-specific regulatory and effector T cells share TCR clonality--possible relation to
747 repetitive CMV infections. Am J Transplant 12 (3):669-681. doi:10.1111/j.1600-6143.2011.03842.x

748 99. Terrazzini N, Bajwa M, Vita S, Cheek E, Thomas D, Seddiki N, Smith H, Kern F (2014) A novel
749 cytomegalovirus-induced regulatory-type T-cell subset increases in size during older life and links
750 virus-specific immunity to vascular pathology. J Infect Dis 209 (9):1382-1392.
751 doi:10.1093/infdis/jit576

752 100. Tovar-Salazar A, Patterson-Bartlett J, Jesser R, Weinberg A (2010) Regulatory function of
753 cytomegalovirus-specific CD4+CD27-CD28- T cells. Virology 398 (2):158-167.
754 doi:10.1016/j.virol.2009.11.038

755 101. Furui Y, Satake M, Hoshi Y, Uchida S, Suzuki K, Tadokoro K (2013) Cytomegalovirus (CMV)
756 seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly
757 donors. Transfusion 53 (10):2190-2197. doi:10.1111/trf.12390

758 102. Poole E, Juss JK, Krishna B, Herre J, Chilvers ER, Sinclair J (2015) Alveolar Macrophages
759 Isolated Directly From Human Cytomegalovirus (HCMV)-Seropositive Individuals Are Sites of
760 HCMV Reactivation In Vivo. J Infect Dis 211 (12):1936-1942. doi:10.1093/infdis/jiu837

761 103. Remmerswaal EB, Havenith SH, Idu MM, van Leeuwen EM, van Donselaar KA, Ten Brinke A,
762 van der Bom-Baylon N, Bemelman FJ, van Lier RA, Ten Berge IJ (2012) Human virus-specific
763 effector-type T cells accumulate in blood but not in lymph nodes. Blood 119 (7):1702-1712.
764 doi:10.1182/blood-2011-09-381574

765 104. Remmerswaal EB, Klarenbeek PL, Alves NL, Doorenspleet ME, van Schaik BD, Esveldt RE,
766 Idu MM, van Leeuwen EM, van der Bom-Baylon N, van Kampen AH, Koch SD, Pircher H,
767 Bemelman FJ, Ten Brinke A, Baas F, Ten Berge IJ, van Lier RA, de Vries N (2015) Clonal
768 evolution of CD8+ T cell responses against latent viruses: relationship among phenotype,
769 localization, and function. J Virol 89 (1):568-580. doi:10.1128/JVI.02003-14

770 105. Woon HG, Braun A, Li J, Smith C, Edwards J, Sierro F, Feng CG, Khanna R, Elliot M, Bell A,
771 Hislop AD, Tangye SG, Rickinson AB, Gebhardt T, Britton WJ, Palendira U (2016)
772 Compartmentalization of Total and Virus-Specific Tissue-Resident Memory CD8+ T Cells in
773 Human Lymphoid Organs. PLoS Pathog 12 (8):e1005799. doi:10.1371/journal.ppat.1005799

774 106. Palendira U, Chinn R, Raza W, Piper K, Pratt G, Machado L, Bell A, Khan N, Hislop AD,
775 Steyn R, Rickinson AB, Buckley CD, Moss P (2008) Selective accumulation of virus-specific
776 CD8(+) T cells with unique homing phenotype within the human bone marrow. Blood 112 (8):3293-
777 3302. doi:10.1182/blood-2008-02-138040

778 107. Gordon CL, Miron M, Thome JJ, Matsuoka N, Weiner J, Rak MA, Igarashi S, Granot T, Lerner
779 H, Goodrum F, Farber DL (2017) Tissue reservoirs of antiviral T cell immunity in persistent human
780 CMV infection. J Exp Med 214 (3):651-667. doi:10.1084/jem.20160758

781 108. Chen KC, Stanton RJ, Banat JJ, Wills MR (2016) Leukocyte Immunoglobulin-Like Receptor 1-
782 Expressing Human Natural Killer Cell Subsets Differentially Recognize Isolates of Human
783 Cytomegalovirus through the Viral Major Histocompatibility Complex Class I Homolog UL18. J Virol
784 90 (6):3123-3137. doi:10.1128/JVI.02614-15

785

786

787 FIGURE LEGENDS

788 Figure 1 – CD4+ and CD8+ T cell IFN-γ responses to pp65, gB, IE1 and IE2 HCMV proteins

789 The T cell IFN-γ responses measured by fluorospot versus donor age from the ARIA study [23] to
790 four lytic expressed HCMV proteins pp65 (green), gB (light blue), IE1 (dark blue) and IE2 (purple)
791 are shown. There is no significant correlation (Spearman rank correlation test) between the
792 magnitude of the IFN-γ response with donor age for any of the four proteins shown.

793 Figure 2 – CMV IgG response related to Age and Latent CMV load in CD14+ monocytes.

794 Serum HCMV IgG levels [immune system ratio (ISR)] from the ARIA study [23] related to donor
795 age (A); there is no significant correlation (Spearman rank correlation test). Latent CMV Load in
796 CD14+ monocytes measured by droplet digital PCR as previously described [23] is shown related
797 to low (<3.5 ISR), medium (3.5 – 4.99 ISR) and high (>5 ISR) CMV IgG levels (B); there is no
798 significant difference in the magnitude of the latent viral load between the three groups (Kruskall-
799 Wallis ANOVA test and Dunn's multiple comparison post-test).

800 Figure 3 – CD4+ T cell IL-10 responses to UL138, LUNA, US28, vIL-10, US3 and pp71 HCMV
801 proteins.

802 The CD4+ T cell IL-10 responses measured by fluorospot versus donor age from the ARIA study
803 [23] to six HCMV proteins UL138 (turquoise circles), LUNA (turquoise squares), US28 (blue
804 triangles), vIL-10 (blue inverted triangles), US3 (green diamonds) and pp71 (orange hexagons) are
805 shown. There is no significant correlation (Spearman rank correlation test) between the magnitude
806 of the IL-10 response with donor age for any of the six proteins shown.

807 Figure 4 – CD8+ T cell IFN-γ responses and CD4+ T cell IFN-γ and IL-10 responses in paired
808 peripheral blood and Bone Marrow samples.

809 IFN-γ and IL-10 T cell responses to 11 HCMV proteins were measured by fluorospot as previously
810 described [23] in a peripheral blood and bone marrow sample from the same donor. The CD8+ T
811 cell IFN-γ (top graph – pink bars), the CD4+ T cell IFN-γ (middle graph – blue bars) and the CD4+
812 T cell IL-10 (bottom graph – turquoise bars) responses in the two compartments are shown.

813 Figure 5 – Illustration of the viral dissemination assay model system.

814 A schematic of the generation of autologous primary dermal fibroblasts from a 2mm punch skin
815 biopsy sample and a microscope image (x10 magnification - bright field) of the fibroblasts growing
816 from a portion of the skin biopsy are shown (A). A pictorial representation of the experimental
817 protocols of the viral dissemination assay (B) and the myeloid cell based modified viral
818 dissemination assay (C) are shown.

Fig. 1

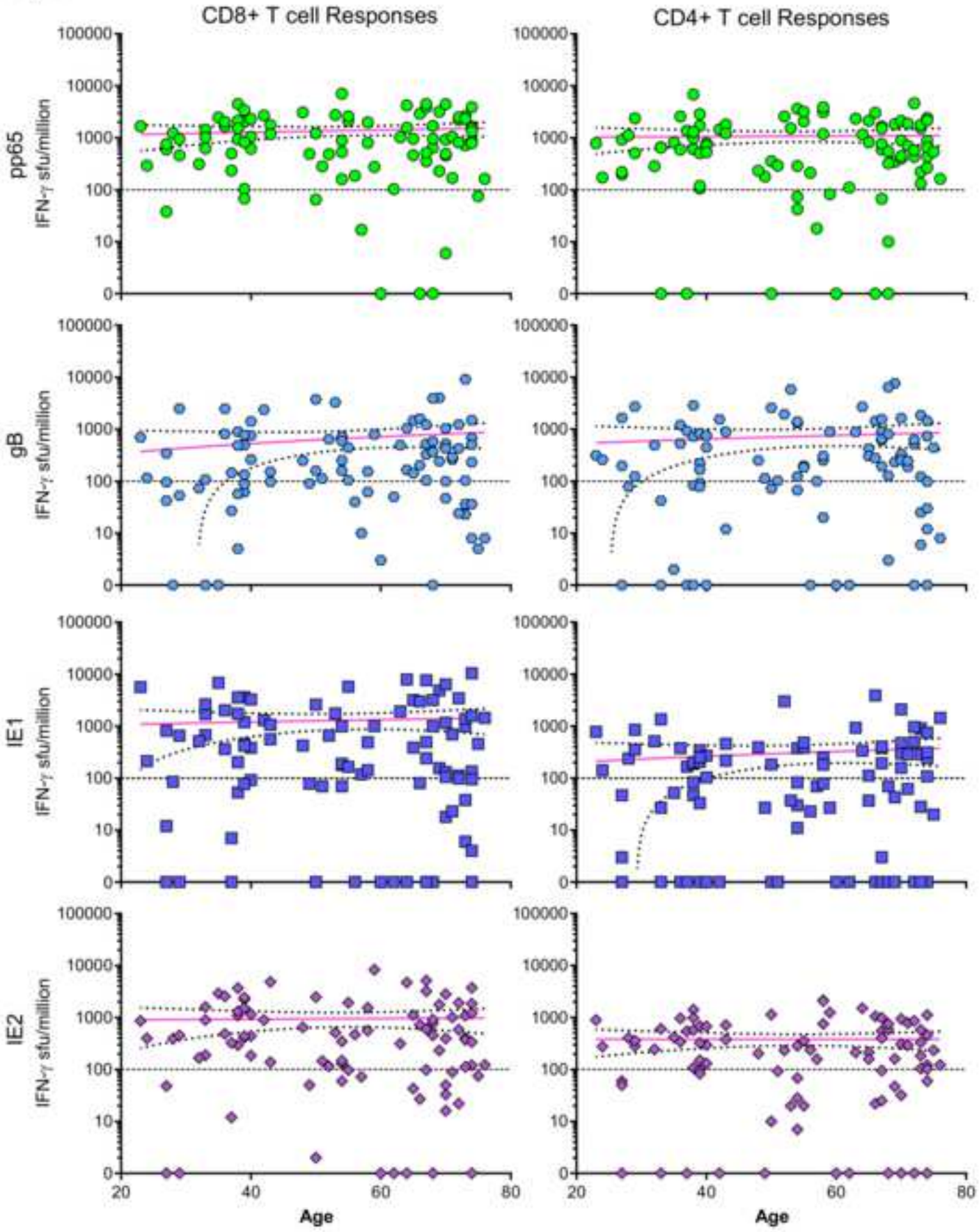


Fig. 2

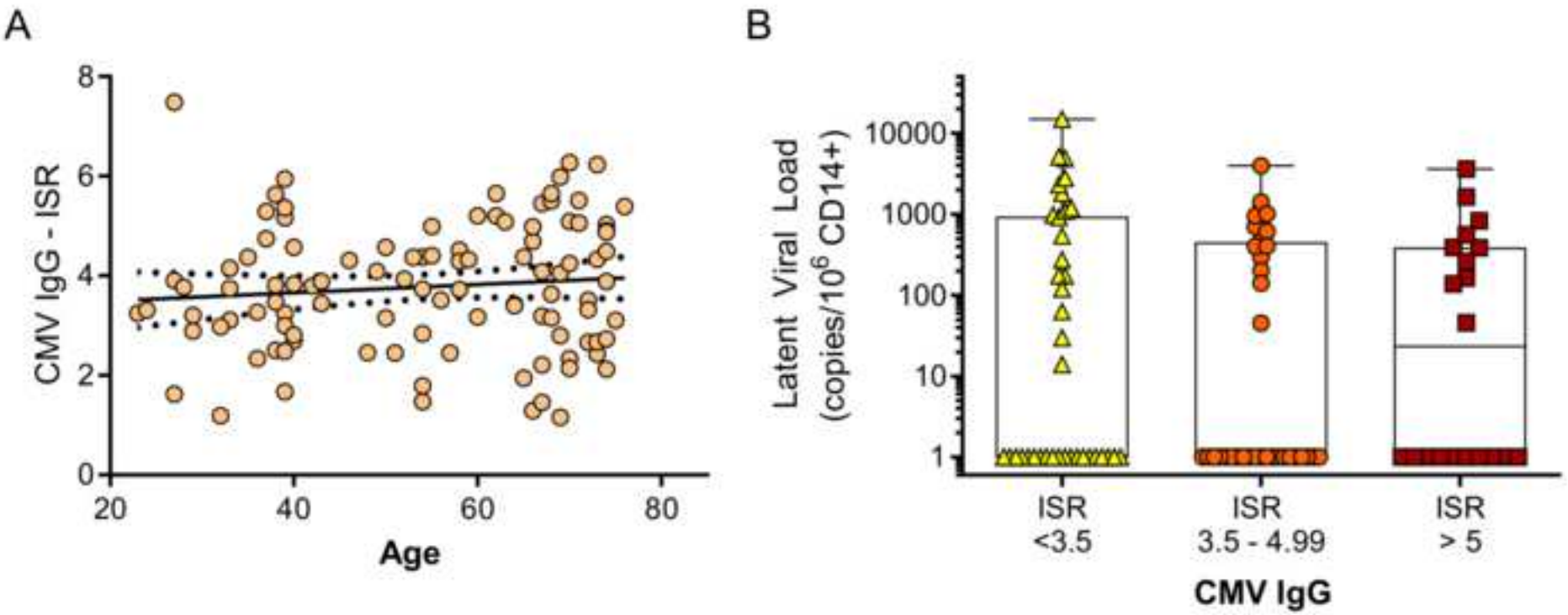


Fig. 3

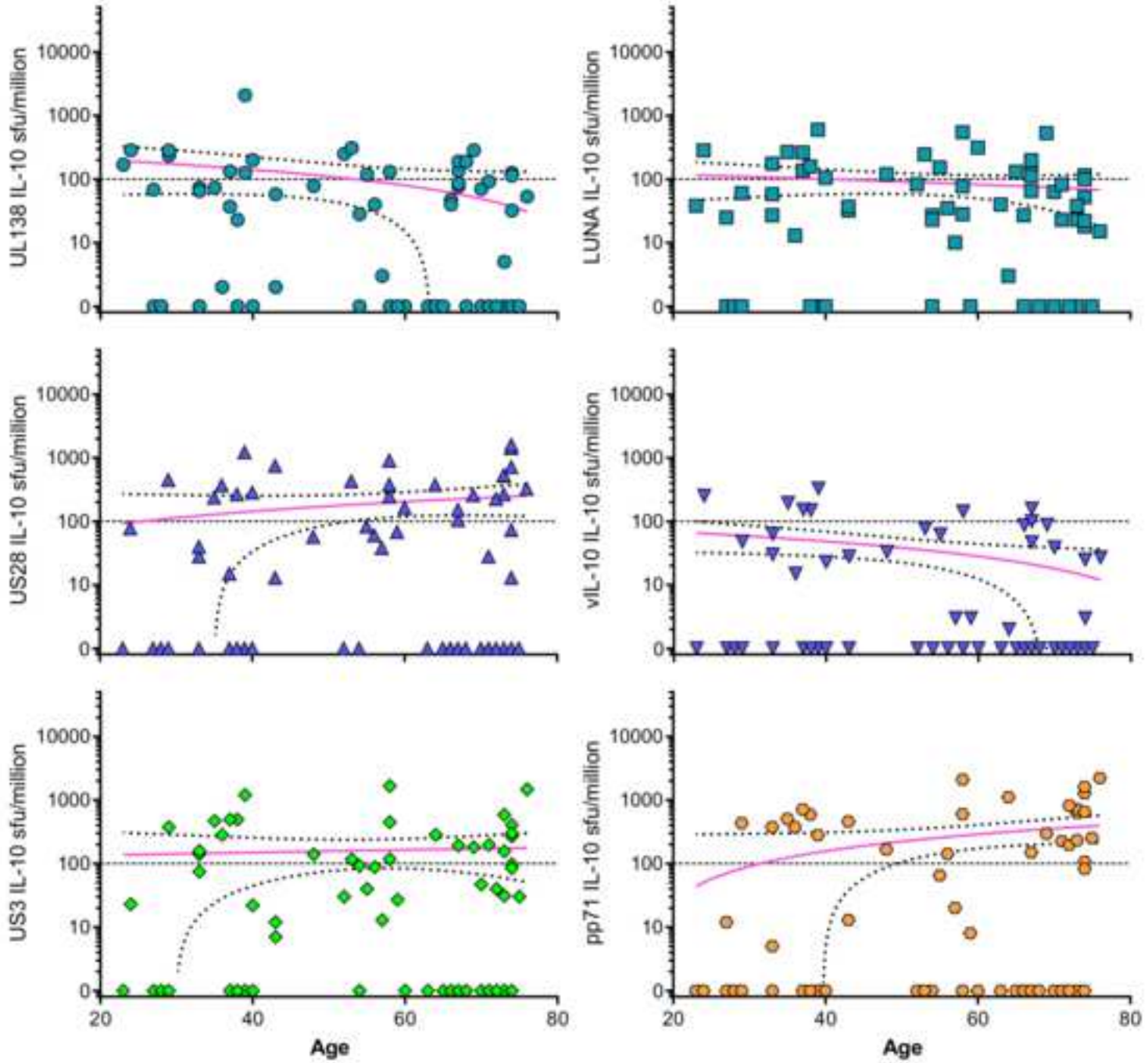


Fig. 4

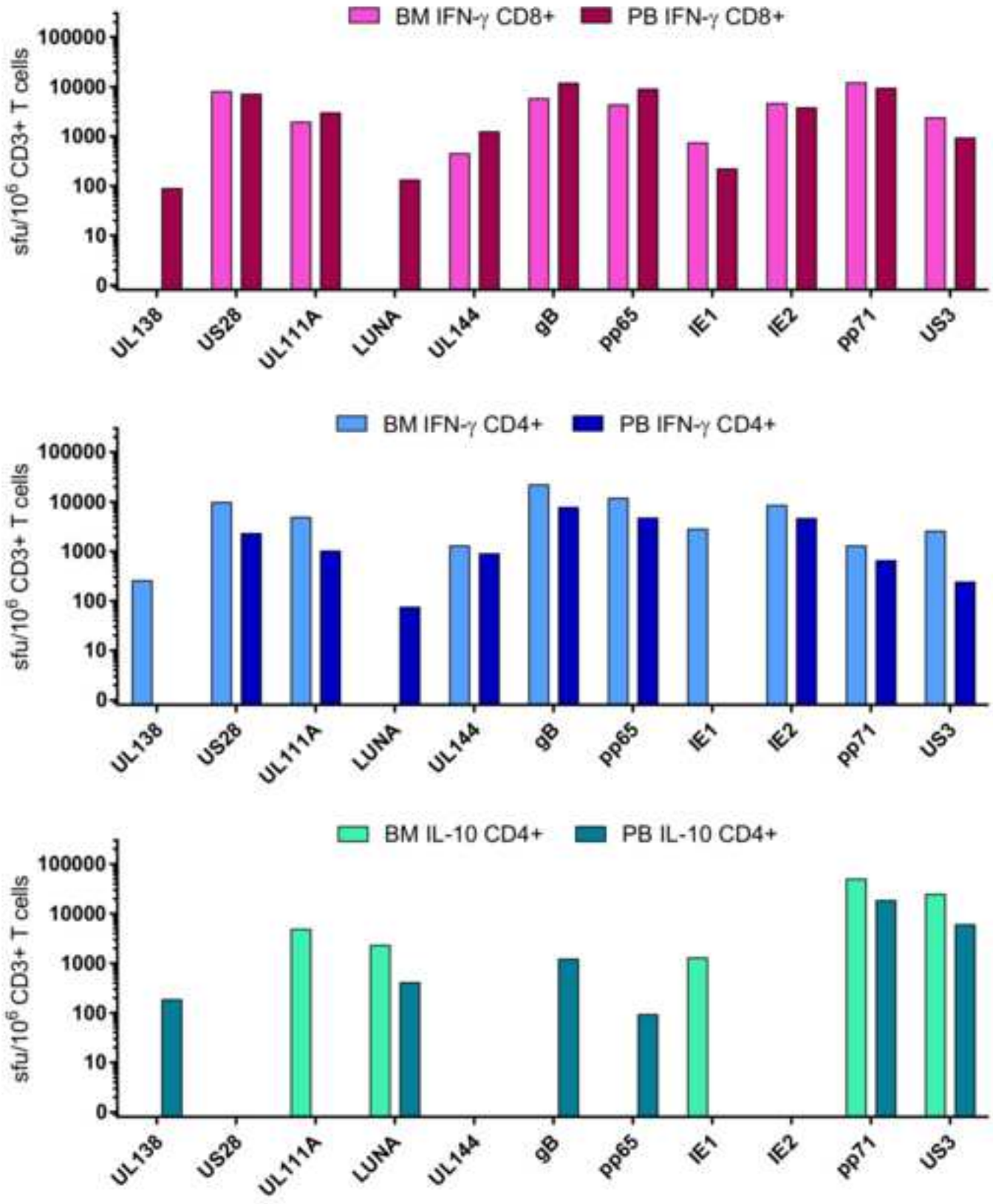


Fig. 5